

## PHYLOGENETIC ANALYSIS OF PHALANGIDA (ARACHNIDA, OPILIONES) USING TWO NUCLEAR PROTEIN-ENCODING GENES SUPPORTS MONOPHYLY OF PALPATORES

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**ABSTRACT.** Recent phylogenetic studies of Opiliones have shown that Cyphophthalmi and Phalangida (= Palpatores + Laniatores) are sister groups, but higher relationships within Phalangida remain controversial. Current debate focuses on whether Palpatores (= Caddoidea + Phalangioida + Ischyropsalidoidea + Troguloidea) is monophyletic or paraphyletic, with Ischyropsalidoidea + Troguloidea (= Dyspnoi) being more closely related to Laniatores. The latter hypothesis was favored in recent combined studies of ribosomal DNA and morphology. Here higher relationships within Phalangida are examined using two nuclear protein-encoding genes, elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) and RNA polymerase II (Pol II), from 27 opilion species representing seven superfamilies. Cyphophthalmi was used as the outgroup. Nucleotide and inferred amino acid sequences were analyzed using maximum-parsimony and maximum-likelihood methods. All analyses recovered Palpatores as the monophyletic sister group to Laniatores with moderate to strong empirical support. Most palpatorean superfamilies were also recovered, but relationships among them were ambiguous or weakly supported. A monophyletic Palpatores was also obtained when EF-1 $\alpha$  and Pol II sequences were analyzed together with 18S and 28S rDNA sequences.

**Keywords:** Molecular systematics, elongation factor-1 $\alpha$ , RNA polymerase II, Opiliones

Members of the arachnid order Opiliones (harvestmen, shepherd spiders, daddy long-legs, etc.) are abundant and often highly visible members of many terrestrial ecosystems. The group is estimated to encompass about 5000 species (Shear 1982). The basic biology of most opilions is unexplored; but the order is known to encompass substantial behavioral and morphological diversity, with much recent work focusing on the structure and evolution of mating systems (e.g., Macías-Ordóñez 1997; Martens 1993; Mora 1987, 1990, 1991; Ramires & Giaretta 1994) and the evolutionary morphology of genitalia (e.g., Martens 1976, 1980, 1986; Martens, Hoheisel & Götze 1981; Hoheisel 1980; Shultz 1998). An understanding of the phylogenetic relationships within Opiliones, and between Opiliones and other arachnid orders, is pivotal to further progress in these and other fields of arachnological research. However, many relationships are unclear and the focus of ongoing investigation and debate (e.g., Giribet et al. 1995, 1999; Giribet & Wheeler 1999; Shultz 1998, 2000).

The goal of the present study is to reconstruct phylogenetic relationships among major lineages within Opiliones using nucleotide and inferred amino-acid sequences from two nuclear protein-encoding genes, elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) and the largest subunit of RNA polymerase II (Pol II).

The monophyly of Opiliones is well established (Weygoldt & Paulus 1979; Shultz 1990), and three principal opilion groups are widely recognized, namely, Cyphophthalmi, Palpatores and Laniatores (Hansen & Sørensen 1904; Shear 1982). Cyphophthalmi (= Sironoidea) is a group of tiny (< 5 mm), hard-bodied and somewhat mite-like opilions characterized by many unique traits, such as a short male genital organ (spermatopositor), elevated ozopores (ozophores), a specialized spine on leg IV of males (adenostyle) and absence of a genital operculum (Shear 1982; Shultz 1998). Laniatores is a species-rich group of heavily sclerotized opilions that have radiated extensively in the neotropics and southeastern Asia (Shear 1982). Members of

this undisputedly monophyletic group are characterized by large, often raptorial palps, unsegmented ovipositors and other unique features. Palpatores is a morphologically diverse assemblage of four superfamilies (Caddoidea, Ischyropsalidoidea, Phalangioida, Troguloidea) united by few morphological synapomorphies. Caddoidea and Phalangioida include the typical "daddy longlegs" familiar to inhabitants of northern temperate regions and are characterized, in part, by well-developed coxal lobes (coxapophyses) and segmented ovipositors. Ischyropsalidoidea and Troguloidea are less familiar groups but encompass a substantial range of morphological diversity. They are characterized, in part, by reduced or absent coxapophyses, diaphanous cheliceral teeth and unsegmented ovipositors.

Opilion systematists have long debated the phylogenetic relationships of the three principal opilion lineages. Šilhavý (1961) placed Cyphophthalmi and Palpatores together as the sister group to Laniatores on the basis of the arrangement of tarsal claws; that is, claws of all legs are similar in Cyphophthalmi and Palpatores, but those of legs I and II differ from those of legs III and IV in Laniatores. A similar but more well-defended system was proposed by Martens and co-workers (Martens 1976, 1980, 1986; Hoheisel 1980; Martens et al. 1981). They also united Cyphophthalmi and Palpatores (= Cyphopalpatores) but argued that the palpatorean superfamily Troguloidea was the sister group to a clade comprising Cyphophthalmi and the remaining Palpatores, a system that rendered Palpatores a paraphyletic group. Their Cyphopalpatores hypothesis was based largely on original morphological studies of the ovipositor and penis, character systems that are uniquely derived in Opiliones. Consequently, the root and branching pattern of the tree proposed by Martens et al. was based on speculative character transformation series rather than inferences derived from outgroups. Shultz (1998) assessed the Cyphopalpatores concept through a parsimony-based morphological analysis of genitalic and non-genitalic characters using a generic taxon sample similar to that of Martens et al. and outgroups (i.e., scorpions and xiphosurans) to polarize non-genitalic characters. The results supported Cyphophthalmi as the sister group to Laniatores + Palpatores (= Phalan-

gida). Phalangida has also been supported by sperm ultrastructure (Juberthie & Manier 1978), arrangement of extrinsic pharyngeal muscles (Shultz 2000) and, especially, molecular sequence data. Giribet et al. (1999) using 18S and 28S ribosomal DNA and Shultz & Regier (unpublished) using EF-1 $\alpha$  and Pol II amino acid sequences have recovered both Cyphophthalmi and Phalangida as monophyletic sister clades.

Recent morphological and molecular analyses appear to have established Phalangida as a monophyletic group, but there is debate concerning basal relationships within this group. These opilions are traditionally divided into the Palpatores and Laniatores, with Palpatores often divided into Dyspnoi (= Ischyropsalidoidea + Troguloidea) and Eupnoi (= Caddoidea + Phalangioida) (Hansen & Sørensen 1904; Šilhavý 1961; Juberthie & Manier 1978; Shultz 1998). However, Giribet et al. (1999) conducted a phylogenetic analysis of Opiliones using 18S and 28S ribosomal DNA sequences and recovered two topologies, one favoring a monophyletic Palpatores and one favoring a paraphyletic Palpatores, with Dyspnoi being the sister group to Laniatores. The molecular characters alone did not strongly favor one hypothesis over the other, but a morphology-based analysis and the combined molecular-morphological study tended to support the Dyspnoi + Laniatores hypothesis. In a subsequent study, Giribet & Wheeler (1999) explored the significance of indels on the phylogenetic analysis of Opiliones. Using 18S ribosomal DNA sequences and morphological characters from Giribet et al. (1999), Giribet & Wheeler recovered Dyspnoi + Laniatores. However, given their exclusion of the 28S rDNA and use of a problematic morphology matrix (see Methods for details), it is probably best to regard Giribet & Wheeler's contribution as a demonstration of a new analytical method rather than a study of opilion relationships.

The present study examines higher relationships within Phalangida using nucleotide and amino-acid sequences from EF-1 $\alpha$  and Pol II with the specific aim of testing the Dyspnoi + Laniatores hypothesis. Sequences were obtained from 27 opilion taxa representing the major opilion superfamilies (i.e., Sironoidea, Travunioidea, Gonyleptoidea, Phalangioida, Caddoidea, Ischyropsalidoidea and Troguloi-

dea). Maximum-parsimony and maximum-likelihood analyses strongly and consistently recovered Palpatores and Laniatores as monophyletic sister groups under all character partitions, weighting schemes and analytical methods. This strong support for the monophyly of Palpatores contrasts with the ambiguous or weakly supported conclusions derived from studies of 18S and 28S ribosomal DNA and morphology (Giribet et al. 1999; Giribet & Wheeler 1999) or morphology alone (Shultz 1998). The represented superfamilies except Travunioidea also tended to be recovered, although support for Ischyropsalidoidea was weak, and it was not possible to conclude whether Dyspnoi and Eupnoi are monophyletic sister groups. Combined analysis of EF-1 $\alpha$  and Pol II sequences with the 18S and 28S rDNA sequences of Giribet et al. (1999) also consistently recovered a monophyletic Palpatores. We regard these results as compelling evidence in support of the Palpatores hypothesis and against the Dyspnoi + Laniatores hypothesis. We suggest that future work on higher-level relationships within Opiliones focus on assessing the monophyly and relationships of superfamilies within Palpatores and Laniatores.

## METHODS

**Abbreviations.**—Abbreviations used in the present study are as follows: bp, base pairs; nt1, first codon position; nt1noLR, nt1 data subset in which any characters that encode a leucine or arginine residue for any taxon are excluded at the homologous position in all taxa; nt1LR, nt1 data subset which includes any characters that encode a leucine or arginine residue for any taxon plus all other homologous characters in other taxa; nt2, second codon position; nt3, third codon position.

**Terminal taxa and sequences.**—Sequences of elongation factor-1 $\alpha$  (EF-1 $\alpha$ : 1092 bp) and the largest subunit of RNA polymerase II (Pol II: 1038 bp) were generated from 27 opilion species representing seven superfamilies. Specimens were collected alive, killed by immersion in 100% ethanol, and stored in 100% ethanol at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ . Voucher specimens will be deposited in the U.S. National Museum of Natural History (Smithsonian Institution, Washington, DC). The list of specimens follows with GenBank accession numbers for EF-1 $\alpha$  and Pol II, respectively, in

parentheses: Sironoidea: *Parasiro coiffaiti* Juberthie 1956 (Sironidae) (AF240852; AF241025 - AF241027), *Siro acaroides* (Ewing 1923) (Sironidae) (AF240855; AF241034 - AF241036). Travunioidea: *Equitius doriae* Simon 1880 (Triaenonychidae) (AF240867; AF241068 - AF241070), *Sclerobunus robustus* (Packard 1877) (Triaenonychidae) (AF240858; AF241042 - AF241044). Gonyleptoidea: *Gonyleptes fragilis* Mello-Leitão 1922 (Gonyleptidae: Gonyleptinae) (AF240868; AF241071 - AF241073), *Progonyleptoidellus striatus* (Roewer 1913) (Gonyleptidae: Progonyleptoidellinae) (AF240873; AF241086 - AF241088), *Sodreana sodreana* (Mello-Leitão 1922) (Gonyleptidae, Sodreaninae) (AF240879; AF241104 - AF241106), *Promitobates ornatus* (Mello-Leitão 1922) (Gonyleptidae, Mitobatinae) (AF240876; AF241095 - AF241097), *Discocyrtus areolatus* Piza 1938 (Gonyleptidae, Pachylinae) (AF240842; AF240994 - AF240996), *Laneosoares inermis* (B. Soares 1944) (Tricommatidae) (AF240871; AF241080 - AF241082), *Pseudobiantes japonicus* Hirst 1911 (Phalangodidae) (AF240874; AF241089 - AF241091), *Proscotolemon sauteri* Roewer 1916 (Phalangodidae) (AF240872; AF241083 - AF241085), *Scotolemon lespesi* (Lucas 1860) (Phalangodidae) (AF240878; AF241101 - AF241103). Caddoidea: *Caddo agilis* Banks 1892 (Caddidae) (AF240838; AF240980 - AF240982), *Caddo pepperella* Shear 1975 (Caddidae) (AF240863; AF241056 - AF241058). Phalangioidea: *Astrobus grallator* Simon 1879 (Sclerosomatidae) (AF240862; AF241053 - AF241055), *Odiellus pictus* (Wood 1868) (Phalangiidae) (AF240850; AF241019 - AF241021), *Phalangium opilio* Linnaeus 1758 (Phalangiidae) (AF240875; AF241092 - AF241094). Ischyropsalidoidea: *Ceratolasma tricantha* Goodnight & Goodnight 1942 (Ceratolasmatidae) (AF240864; AF241059 - AF241061), *Hesperonemastoma modestum* (Banks, 1894) (Ceratolasmatidae) (AF240869; AF241074 - AF241076), *Ischyropsalis luteipes* Simon 1872 (Ischyropsalididae) (AF240870; AF241077 - AF241079), *Sabacon imamurai* Suzuki 1964 (Sabaconidae) (AF240877; AF241098 - AF241100), *Taracus pallipes* Banks 1894 (Sabaconidae) (AF240881; AF241110 - AF241112). Troguloidea: *Di-*

*cranolasma scabrum* (Herbst 1799) (Dicranolasmatidae) (AF240866; AF241065 - AF241067), *Trogulus nepaeformis* (Scopoli 1763) (Trogulidae) (AF240880; AF241107 - AF241109), *Nipponopsalis abei* (Sato & Suzuki 1939) (Nipponopsalididae) (AF137391; AF138993 - AF138995), *Dendrolasma dentipalpe* Shear & Gruber 1983 (Nemastomatiidae) (AF240865; AF241062 - AF241064).

**Outgroups and phylogenetic framework.**—The present study focuses on higher-level relationships within Phalangida and thus uses Cyphophthalmi as the outgroup. As summarized in the introduction, Opiliones is an unambiguously monophyletic group and recent phylogenetic analyses strongly support Cyphophthalmi and Phalangida as monophyletic sister groups. Results from molecular studies have been particularly convincing. Giribet et al. (1999) conducted an analysis of higher relationships within Opiliones and outgroups (i.e., Ricinulei, Solifugae, Scorpiones, Xiphosura) using 18S and partial 28S ribosomal DNA sequences and consistently recovered Cyphophthalmi and Phalangida as sister groups. Shultz & Regier (unpublished) used EF-1 $\alpha$  and Pol II in a broad study of ordinal relationships within Arachnida and reconstructed Cyphophthalmi and Phalangida as monophyletic clades with high bootstrap support. Opiliones was rooted in the analysis with 26 outgroup species representing Xiphosura and all arachnid orders except Palpigradi. Taken together, these results effectively falsify the Cyphopalpatores hypothesis and strongly support the monophyly of Phalangida. It would have been possible in the present study to include all or some non-opilion arachnids, but these were excluded because inclusion of certain rapidly evolving arachnid lineages (especially Acari and Pseudoscorpiones) destabilizes even well-established relationships within Opiliones. We have therefore used the two species from Sironoidea (Cyphophthalmi) to root Phalangida.

**Sequence data.**—Procedures to generate sequence data sets have been published (Regier & Shultz 1997). In brief, total nucleic acids were isolated; cDNA copies of EF-1 $\alpha$  and Pol II mRNA were reverse transcribed; dsDNA copies were amplified by PCR and subsequently gel isolated; the resulting PCR fragments were used as templates for another round of PCR amplification with nested prim-

ers; the resulting fragments were gel isolated and sequenced. If the resulting fragment concentration was too low to sequence directly, it was either concentrated or reamplified with M13 sequences present at the 5' ends of all primer sequences. The same M13 sequences were also used as primers for thermal cycle/dideoxy sequencing. Sequencing reactions were fractionated and preliminary analyses were performed with Perkin-Elmer/ABI automated DNA sequencers. Automated DNA sequencer chromatograms were edited and contigs were assembled using the pregap and gap4 programs within the Staden software package (Staden et al. 1999). Sequences from different species were aligned and Nexus-formatted nucleotide data sets were constructed using the Genetic Data Environment software package (version 2.2, Smith et al. 1994). All sequences lacked indels. Amino acid data sets were inferred from nucleotide sequences using the universal nuclear genetic code option in MacClade, version 3.08 (Maddison & Maddison 1992).

**Data analysis.**—Maximum parsimony (MP) analyses of nucleotide and amino acid data sets were performed in PAUP\*4.0 (Swofford 1998) using unordered, equally weighted characters. The following data sets were analyzed: all-nucleotide, nt1 + nt2, nt1noLR + nt2, nt3, amino acids. Amino acids were also analyzed using a "Protpars" step matrix constructed in MacClade. The step matrix consisted of transformation scores of "1" or "2" determined by the minimum number of non-synonymous nucleotide changes separating particular codons. Serine codons that differed at nt1 were coded separately (termed "non-disjunct" in MacClade). Analysis consisted of a heuristic search using TBR branch swapping with random taxon addition (100 replications). Bootstrap values (Felsenstein 1985) were also obtained using heuristic searches (1000 replications each with 10 random-sequence addition replicates). The incongruence length difference test (Farris et al. 1995), implemented in PAUP\* 4.0 as the partition homogeneity test (1000 replications), was used to test for the significance of conflict between genes and character partitions.

Maximum likelihood (ML) analyses of nucleotide data sets were performed with PAUP\* 4.0 under the optimal GTR model of sequence evolution, the optimal model based on a series

of likelihood-ratio tests conducted according to Swofford et al. (1996: 430–438; see also Shultz & Regier 2000). Among-site rate variation was accommodated within the GTR model by likelihood estimations of separate rates for individual codon positions by gene and by fitting total likelihood-estimated character change to a gamma distribution with invariable sites estimated separately (Hasegawa, Kishino & Yano 1985). We call the former model GTR-ssr and the latter GTR +  $\Gamma$  + I. The GTR-ssr model was applied to total nucleotide data and the GTR +  $\Gamma$  + I model to the nt1 + nt2 and the nt1noLR + nt2 data sets.

As a first step in our exploration of tree space using ML analysis, we used an MP tree derived from analysis of amino acids as the input topology on which likelihood parameters were optimized. NNI branch swapping was then performed, and new likelihood parameters were estimated from the most likely topology. TBR branch swapping was conducted on the new tree and likelihood parameters were re-estimated. These parameters were then used as input for a heuristic search with NNI branch swapping and 100 random taxon additions. The parameters from the overall best tree were re-optimized. Bootstrap analyses were too computationally demanding to be performed in the same manner. Instead, the Neighbor Joining algorithm coupled with an ML-estimated distance matrix were used (1000 replications). For each bootstrapped data set, a ML distance matrix was calculated that assumed a minimum evolution objective function and that used identical parameters (i.e., rate matrix, base frequencies,  $\alpha$  value, proportion of invariant sites) to those estimated from the ML topology of the original data set. Bootstrap values for the nt1 + nt2 and the nt1noLR + nt2 data sets were calculated in this manner.

ML analysis of the amino-acid data set was performed using the protml program within the MOLPHY software package (version 2.2, Adachi & Hasegawa 1994) and the empirical transition matrix compiled by Jones, Taylor & Thornton (1992). All 37681 amino acid parsimony trees within 4 steps of the minimum-length tree (= 354 steps) were read in batch into protml, and the tree with the highest likelihood score was selected. The significance of differences in the fit of individual data sets to

alternative topologies under both MP and ML criteria was assessed by the test of Kishino & Hasegawa (1989), using the “Tree Scores” option as implemented in PAUP\* 4.0. Only fully dichotomous trees were compared; that is, single nodes (e.g., Dyspnoi + Laniatores) were constrained and the remaining set of unconstrained relationships was reoptimized. Percentage differences of all pairwise combinations of EF-1 $\alpha$  and of Pol II amino acid and nt3 data sets were calculated in PAUP\* 4.0. Average differences across the basal node of various groups were calculated by averaging all values across the basal dichotomy within a particular clade. Base compositions were calculated by gene and by codon position type using PAUP\* 4.0.

**Combined analyses with ribosomal DNA.**—Unweighted MP analyses were conducted on data matrices that combined the 18S and 28S ribosomal sequences generated by Giribet et al. (1999) (15 opilions and 5 outgroups) with the EF-1 $\alpha$  and Pol II sequences (27 opilions) generated in the present study. Two combined matrices were constructed. In the first, ribosomal data were combined with complete nucleotide sequences from EF-1 $\alpha$  and Pol II for a total of 4329 characters (1117 parsimony-informative characters), and, in the second, the ribosomal data were combined with amino acids from EF-1 $\alpha$  and Pol II for a total of 2909 characters (346 parsimony-informative characters). In both cases, the ribosomal sequences were aligned and edited as described in Giribet et al. (1999). Both matrices included 35 terminal taxa, which encompassed 15 taxa with both ribosomal and EF-1 $\alpha$  + Pol II sequences, nine taxa with only ribosomal sequences, and 11 taxa with only EF-1 $\alpha$  + Pol II sequences. Regions of the matrix lacking sequence data were treated as missing characters.

The specific strategy for combining ribosomal and protein-encoding sequences is presented below. The following five species were represented by both rDNA and protein-encoding sequences: *Parasiro coiffaiti* (GenBank accession nos.: 18S: U36999; 28S: U91495), *Equitius doriae* (18S: U37003, 28S: U91503), *Scotolemon lespesi* (18S: U37005, 28S: U91506), *Caddo agilis* (18S: U91487, 28S: U91502) and *Ischyropsalis luteipes* (18S: U37000, 28S: U91497). We also combined sequences from five pairs of closely related taxa,

specifically, *Siro rubens* Latreille 1804 (18S: U36998, 28S: U91494) was combined with EF-1 $\alpha$  and Pol II sequences for *Siro acaroides*, *Odiellus troguloides* (Lucas 1847) (18S: X81441, 28S: U91500) was combined with *Odiellus pictus*, *Dicranolasma soerenseni* Thorell 1876 (18S: U37001, 28S: U91498) was combined with *Dicranolasma scabrum*, the nemastomatid *Centetostoma dubium* (Mello-Leitão 1936) (18S: U37002, 28S: U91499) was combined with *Dendrolasma dentipalpe*, and the pachyline gonyleptid *Pachyloides thorelli* Holmberg 1878 (18S: U37007, 28S: U91508) was combined with *Discocyrtus areolatus*. Taxa represented only by rDNA sequences included an unidentified *Stylocellus* Westwood 1874 (18S: U91485, 28S: U91496), an unidentified *Oncopus* Thorell 1876 (18S: U91488, 28S: U91504), *Maioreus randoi* Rambla 1991 (18S: U37004, 28S: U91505), *Gnidia holmbergi* Soerensen 1912 (U37006, 28S: U91507), the solifuge *Eusimonia wunderlichi* Pieper 1977 (18S: U29492, 28S: none), the ricinuleid *Pseudocellus pearsei* (Chamberlin & Ivie 1938) (18S: U91489, 28S: none), the scorpion *Androctonus australis* C.L. Koch 1839 (18S: X77908, 28S: none), and the xiphosurans *Limulus polyphemus* (Linnaeus 1758) (18S: U91490, 28S: U91492) and *Carcinoscorpius rotundicauda* (Latreille 1802) (18S: U91491, 28S: U91493).

Giribet et al. (1999) had also presented a matrix of 45 morphological characters from an unreferenced literature review, but we did not include it in the combined analysis due to numerous ambiguities and inaccuracies. For example, palpal claws were coded as well developed in Sironoidea, but these claws are reduced compared to pedal claws (Hansen & Sørensen 1904; Shultz 1998). Two characters focusing on "fusion of abdominal tergites" were questionable, because males and females were coded separately, thereby making the characters non-independent. Metapeltidial cones were coded as "absent" in *Caddo* but are present (Shultz 1998). Internal longitudinal muscles of the ovipositor were coded as "present" in *Parasiro* and *Siro*; "reduced" in *Odiellus*, *Caddo*, *Dicranolasma*, *Pachyloides*, *Scotolemon* and *Centetostoma* but they are "absent" in sironoids, phalangioids, *Caddo* and *Dicranolasma* and well developed in *Centetostoma*, *Scotolemon* and other gonyleptoids

(Martens et al. 1981). Circular muscles of the ovipositor were coded as absent in *Odiellus* and *Caddo*, but they are present in phalangioids and *Caddo* (Martens et al. 1981). Given concerns about accuracy, the absence of source citations and the need to expand the matrix to include taxa not considered by Giribet et al. (1999), we chose not to include the morphological matrix in the combined study.

## RESULTS

**Pairwise differences and base composition.**—Inferences of phylogenetic relationships among arthropods at taxonomic levels deeper than those in the present study have demonstrated that EF-1 $\alpha$  and Pol II sequences retain phylogenetic signal, although the synonymous changes of nt3 can be very homoplasious due to overlapping substitutions. Across Opiliones, observed pairwise differences at nt2, at which all changes are non-synonymous, did not exceed 8.1% for either gene. By contrast, maximum observed pairwise differences at nt3 were 50.6% for EF-1 $\alpha$  and 70.5% for Pol II, and were never less than 10% for either gene. These observations are consistent with the greater homoplasy at nt3 relative to nt2 (and nt1, in which changes are a mixture of synonymous and non-synonymous changes). For example, fitting nt1, nt2, and nt3 data sets to the topology shown in Fig. 1 resulted in overall Retention Indices of 0.6347, 0.8351, and 0.3825, respectively.

Chi-square tests of homogeneity across taxa revealed no significant heterogeneity ( $P \gg 0.05$ ) in most partitions examined (i.e., nt1, nt2 and nt1noLR of both genes separately and combined), both with and without constant sites excluded. Exceptions were nt1 minus constant sites for Pol II, nt3 for Pol II, and those partitions that included nt3 of Pol II (i.e., all-nucleotides and nt3 combined), all of which showed highly significant base heterogeneity ( $P < 0.0001$ ).

**Phylogenetic analyses of EF-1 $\alpha$  and Pol II.**—Partition homogeneity tests of character partitions revealed no significant inconsistencies in the phylogenetic signals of EF-1 $\alpha$  and Pol II, except in comparisons that included nt3 of Pol II (Table 1). Given these results, and uncertainties concerning the importance and utility of the partition homogeneity tests as an assay of combinability (Baker & DeSalle

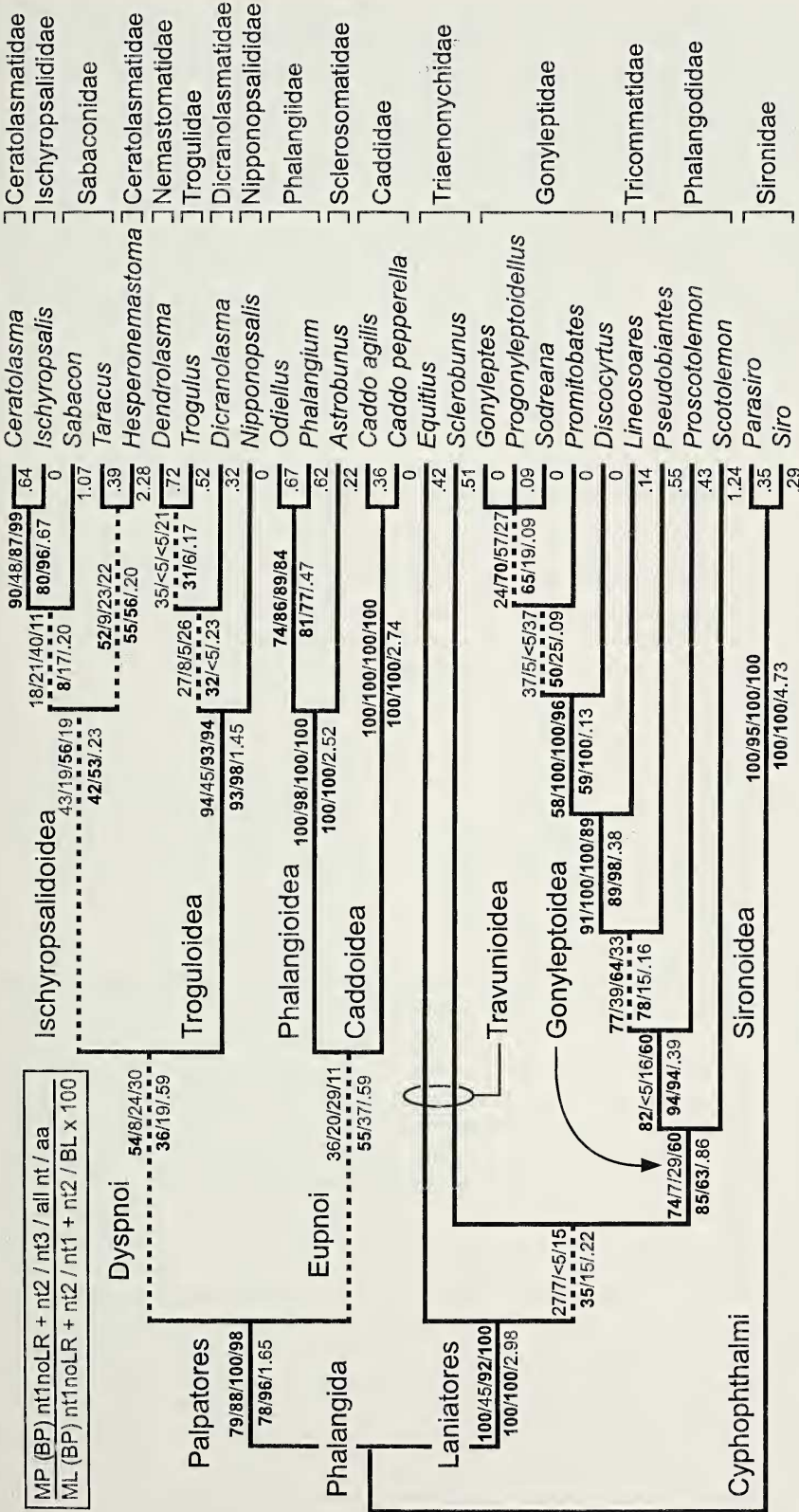


Figure 1.—Phylogenetic tree summarizing results from analyses of elongation factor-1 $\alpha$  and RNA polymerase II for 27 opilion taxa. The depicted topology is the optimal tree derived from maximum-likelihood analysis of nt1noLR + nt2 using the GTR +  $\Gamma$  + I model (see text for details). Branches depicted by solid lines are considered well supported as indicated by empirical support (i.e., bootstrap value), stability and robustness to analytical method. Branches depicted by dashed lines are considered weakly supported by the same criteria. Numbers above internal branches are bootstrap percentages (BP) derived from maximum-parsimony (MP) analyses of nt1noLR + nt 2, nt3, all nucleotides and amino acids, respectively. The first two numbers below internal branches are bootstrap percentages derived from maximum-likelihood (ML) analyses of nt1noLR + nt2 and nt1 + nt2, respectively. The third number below internal branches and the only number under terminal branches are branch lengths (BL) derived from maximum-likelihood analysis of nt1noLR + nt2 multiplied by 100. Numbers in bold type indicate relationships recovered in the strict consensus of maximum parsimony trees or in the tree of highest likelihood.

1995; Cannatella et al. 1998), we performed combined analyses of all partitions.

Maximum-parsimony (MP) analyses of all character partitions (all-nucleotides, nt1 + 2, nt1noLR + nt2, nt3, amino acids, amino-acids-protpars) recovered the following higher clades within their respective minimal-length trees (bootstrap percentages [BP] in parentheses): Phalangida (100, 100, 100, 95, 100, 100); Laniatores (92, 99, 100, 45, 100, 100); Palpatores (100, 96, 79, 88, 87, 76); Troguloidea (93, 97, 94, 45, 94, 97); Caddoidea (100, 100, 100, 100, 100, 100) and Phalangioida (100, 100, 100, 98, 100, 100). Ischyropsalidoidea was recovered by all-nucleotides (BP 56), nt1 + nt2 (BP 73), nt1noLR + nt2 (BP 43) and amino-acids-protpars (BP 30) but was recovered as a paraphyletic group by nt3 and amino acids. Dyspnoi (= Troguloidea + Ischyropsalidoidea) was recovered by all-nucleotides (BP 24), nt1 + nt2, (BP 59), nt1noLR + nt2 (BP 54), and amino-acid-protpars (BP 47). Eupnoi (= Caddoidea + Phalangioida) was recovered by nt1noLR + nt2 (BP 36) within a subset of 20 MP trees. Gonyleptoidea was recovered by all-nucleotides, nt1noLR + nt2, amino acids, and amino-acids-protpars with weak-to-moderate bootstrap support (i.e., 29–74%). Travunioidea was recovered as a paraphyletic group with *Equitius* being the sister group to Gonyleptoidea and/or occurring within Gonyleptoidea.

The all-nucleotide and nt1 + nt2 data sets significantly preferred a monophyletic Palpatores over the MP clade constrained to a monophyletic Laniatores + Dyspnoi (*P* values ranged from 0.015 to 0.028), according to the test of Kishino & Hasegawa (1989); the other data sets were indecisive.

The ML topology recovered by analysis of the nt1noLR + nt2 data set is shown in Fig. 1. All maximum likelihood analyses (three models of nucleotide substitution and four data subsets, see Methods) recovered the following clades in their ML topologies (BP values for analysis of nt1 + nt2 and nt1noLR + nt2, respectively, are in parentheses): Phalangida (100, 100), Laniatores (100, 100), Palpatores (96, 78), Troguloidea (98, 93), Gonyleptoidea (63, 85), Phalangioida (100, 100), Caddoidea (100, 100), and Ischyropsalidoidea (53, 42). Eupnoi (37, 55) was recovered by all three nucleotide data sets. Dyspnoi (19, 36) was recovered only by the nt1noLR + nt2

data set. ML analysis of amino acids recovered Phalangida, Laniatores, Palpatores, Troguloidea, Caddoidea, Phalangioida, and Gonyleptoidea.

**Combined analyses.**—Combined unweighted MP analyses of 18S + 28S rDNA and EF-1 $\alpha$  + Pol II sequences strongly and consistently corroborated the monophyly of Palpatores (Fig. 2), the result favored by analysis of EF-1 $\alpha$  + Pol II alone, not the Dyspnoi + Laniatores hypothesis that tended to be recovered by the rDNA data analyzed alone (Giribet et al. 1999). The strict consensus of 360 trees (length, 6067; CI, 0.31; RI, 0.48) derived from analysis of ribosomal and protein-encoding DNA included Opiliones, Phalangida, Laniatores, Palpatores, Dyspnoi and all represented opilion superfamilies (except Travunioidea) but did not recover Eupnoi (Fig. 2A). The Dyspnoi + Laniatores hypothesis was recovered in only 5% of bootstrap replicates and required 30 additional steps for recovery by parsimony analysis. Strict consensus of 78 trees (length, 865; CI, 0.54; RI, 0.75), derived from MP analysis of ribosomal DNA and EF-1 $\alpha$  + Pol II amino acids included the same major opilion clades listed above but also recovered Eupnoi (Fig. 2B). The Dyspnoi + Laniatores hypothesis was recovered in only 5% of bootstrap replicates and required 10 additional steps for recovery by parsimony.

## DISCUSSION

**Status and future of higher relationships in Opiliones.**—Recent phylogenetic analyses clearly indicate that Opiliones consists of two clades, Cyphophthalmi and Phalangida (Giribet et al. 1999; Shultz 1998; Shultz & Regier unpubl. data). Results from analysis of elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) and RNA polymerase II (Pol II), both alone and combined with 18S and 28S rDNA, strongly support the monophyly of Palpatores and Laniatores and are inconsistent with the recently proposed Dyspnoi + Laniatores hypothesis (Giribet et al. 1999, Giribet & Wheeler 1999). Molecular data examined thus far have consistently recovered three palpatorean superfamilies (Caddoidea, Phalangioida, Troguloidea) as monophyletic groups (Giribet et al. 1999; present study), but monophyly of the remaining superfamily, Ischyropsalidoidea, is more problematic. This superfamily was recovered by



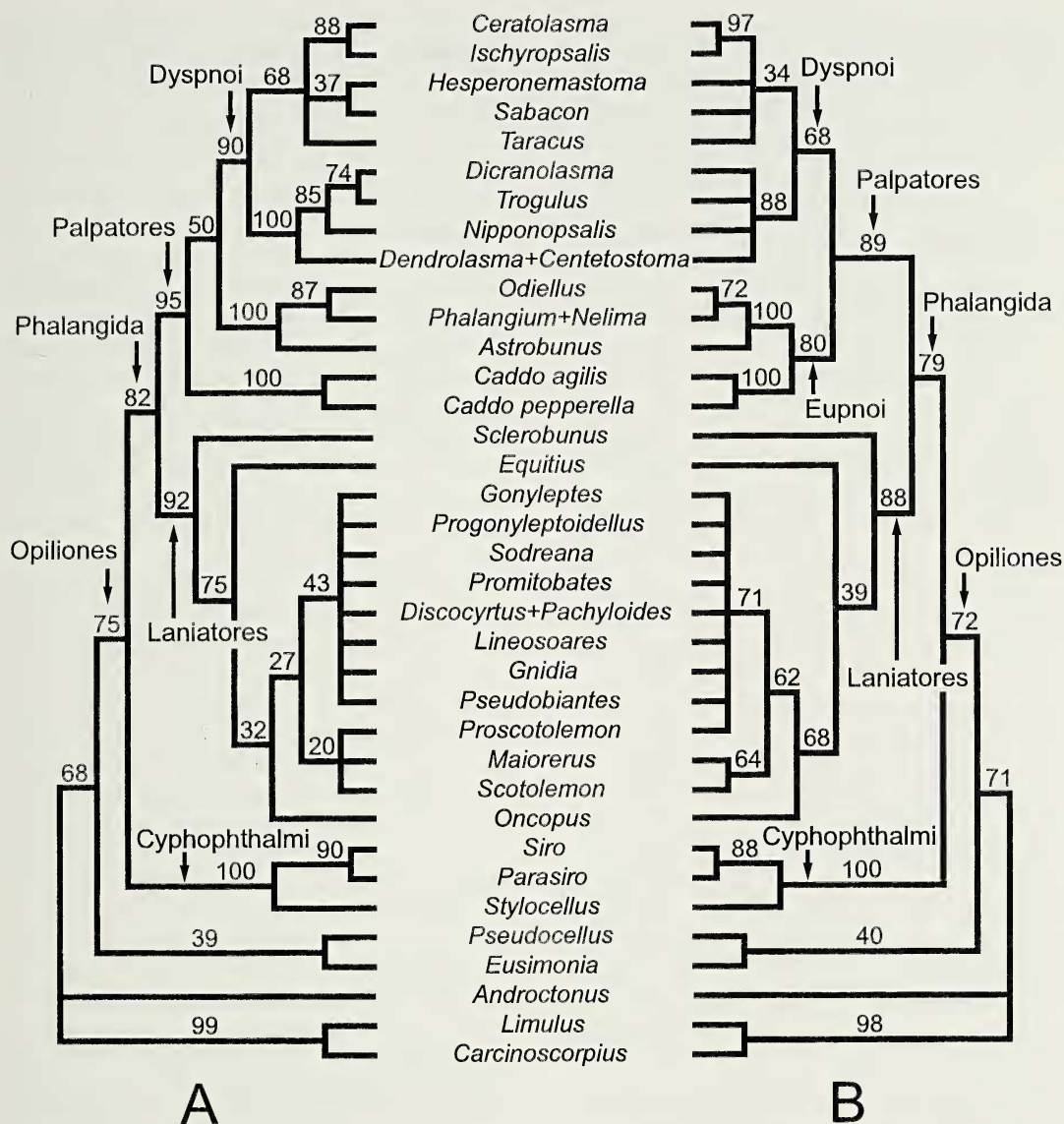


Figure 2.—Strict consensus trees derived from combined, unweighted maximum parsimony analyses of 18S rDNA, 28S rDNA, EF-1 $\alpha$  and Pol II. A. Strict consensus of 360 trees derived from analysis of rDNA and EF-1 $\alpha$  + Pol II nucleotides. B. Strict consensus of 78 trees derived from analysis of rDNA and EF-1 $\alpha$  + Pol II amino acids. Numbers above internal branches are bootstrap percentages.

EF-1 $\alpha$  and Pol II in only a few analyses with low bootstrap support (Fig. 1) and in both combined analyses with moderate support (Fig. 2). The traditional grouping of Caddoidea and Phalangioidea into Eupnoi and Ischyropsalioidea and Troguloidea into Dyspnoi could be neither falsified nor confirmed by EF-1 $\alpha$  and Pol II, but they are both strongly supported in the combined analysis of 18S and 28S rDNA conducted by Giribet et al. (1999).

Based on the present study, it appears that phylogenetic signal within EF-1 $\alpha$  and Pol II can resolve the deepest nodes within opilion phylogeny but become less useful at lower taxonomic levels. Specifically, relationships among and within superfamilies are largely unresolved by these two genes. This suggests that progress in opilion systematics will require the development of new genes, especially those that have evolved at a greater rate

than the two examined here as well as renewed efforts to search for informative morphological characters. Expanded taxon sampling is also important, and particular emphasis should be placed on sampling representatives from basally divergent lineages of each well-defined opilion clade. These include, but are not limited to, *Crosbycus* from Ischyropsalidoidea, neopilionids from Phalangioidea, acropsopilionids from Caddoidea and the travunioid families from Laniatores.

**Relationships within Ischyropsalidoidea.**—Ischyropsalidoidea is a morphologically diverse group encompassing three families (Ischyropsalididae, Sabaconidae, Ceratolasmatidae), but the monophyly of the superfamily is not well established. Shear (1986) united the ischyropsalidoids with four synapomorphies, namely, metapeltidial cones, unsegmented ovipositor, reduced palpal claw and presence of male cheliceral glands. However, metapeltidial cones are also present in Caddoidea (*Caddo*) and the remaining characters occur in some or all members of Troguloidea (Shultz 1998). Further, our molecule-based results do not support Shear's (1986) system of ischyropsalidoid families (Fig. 3A), but neither do they strongly conflict with it. Specifically, our analyses consistently recovered an *Ischyropsalis* + *Ceratolasma* grouping and frequently but weakly placed the ceratolasmatid *Hesperonemastoma* as the sister group to one or both sabaconids (Fig. 1). These results suggest that Ceratolasmatidae and Sabaconidae are not natural groups, although the evidence is weak. Still, para- or polyphyly of the two families is a hypothesis to be tested and is compatible with several other lines of evidence. First, few morphological characters support monophyly of the morphologically diverse Ceratolasmatidae (= *Acuclavella*, *Ceratolasma*, *Crosbycus*, *Hesperonemastoma*). Shear's (1986) diagnosis of the family includes no unique features and most characters have multiple states within the family (e.g., "... pairs of tubercles on scutum ... high and acute, or blunt and appressed ... or absent," "chelicerae with or without glands in males"; "palpi long, with ... or short, without ... plumose setae") (p. 13). Second, monophyly of Sabaconidae can also be questioned, although it is substantially more convincing than Ceratolasmatidae. Potential sabaconid synapomorphies include deep invagination in anterior

midline of carapace, reduced sclerotization, and enlarged palpal tibia and tarsus (Shear 1986). The first of these appears to be unique to the family, but the second seems to depend on a priori suppositions of character transformation. The third character is undoubtedly derived and clearly expressed in *Sabacon*, but is less obvious in *Taracus*, especially when the palps are compared to those of *Hesperonemastoma* (original observations). Thus, Sabaconidae is a probable but not unambiguously demonstrated family. Third, in his re-description of *Ceratolasma*, Gruber (1978) noted two phenetic groupings of taxa within Ischyropsalidoidea that are broadly congruent with our findings. Specifically, *Ceratolasma* and *Ischyropsalis* share a "prominent sternum, large labium, palpi without plumose setae, but with numerous microtrichia, and also a complex midgut anatomy" (p. 109). He also noted that *Hesperonemastoma* is similar to sabaconids in having "less developed sterna, small labia, palpi with extensive development of plumose setae and reduction of the microtrichial cover" and simpler midgut anatomy. Admittedly, both lists are mosaics of primitive and derived traits, and more intensive morphological and molecular analyses are needed to make progress in ischyropsalidoid systematics. The molecular data are open to criticism in that relationships among the relevant taxa are unstable and *Hesperonemastoma* appears to have undergone more rapid molecular evolution than other ischyropsalidoids, which may account for the ambiguity.

**Relationships within Troguloidea.**—In contrast to ischyropsalidoids, the troguloids are a well-defined, monophyletic group. Martens (Martens 1980, 1986; Martens et al. 1981; Martens & Suzuki 1966) and Shear & Gruber (1983) proposed several synapomorphies, including a penis with two longitudinal muscles, unique unsegmented ovipositor, fusion of sternum and leg coxae, clavate palpal setae, and reduced palpal claws. Only the latter is open to question, as reduced palpal claws are also present in ischyropsalidoids. The present study included one representative from each of the four troguloid families (Nipponopsalididae, Nemastomatidae, Dicranolasmatidae, Trogulidae) and our results strongly supported the monophyly of the superfamily under all character partitions and analytical methods (Fig. 1). However, relationships with-

in Troguloidea were ambiguous. Shear & Gruber (1983) regarded Dicranolasmatidae and Nemastomatidae as sister groups on the basis of one character (penis muscles with long tendons) but did not propose relationships between this clade, Trogulidae and Nipponopsalididae. Shultz (1998) proposed dicranolasmatids and trogulids as sister groups based on heavy sclerotization and anteriorly projecting eye tubercle or "hood" in these taxa. Our molecular data do not strongly support any phylogenetic arrangement among the troguloid families, although there was a tendency to recover the nemastomatid "Dendrolasma" as the sister group to the remaining representatives.

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